

# Cytochrome P450-catalyzed hydroxylation of taxa-4(5),11(12)-diene to taxa-4(20),11(12)-dien-5 $\alpha$ -ol: the first oxygenation step in taxol biosynthesis

Jerry Hefner<sup>1</sup>, Steven M Rubenstein<sup>2</sup>, Raymond EB Ketchum<sup>3</sup>, Donna M Gibson<sup>3</sup>, Robert M Williams<sup>2</sup> and Rodney Croteau<sup>1</sup>

**Background:** The structural complexity of taxol dictates continued reliance on biological production methods, which may be improved by a detailed understanding of taxol biosynthesis, especially the rate-limiting steps. The biosynthesis of taxol involves the cyclization of the common isoprenoid intermediate geranylgeranyl diphosphate to taxa-4(5),11(12)-diene followed by extensive, largely oxidative, modification of this diterpene olefin. We set out to define the first oxygenation step in taxol biosynthesis.

**Results:** Microsomal enzymes from *Taxus* stem and cultured cells were used to define the first hydroxylation of taxadiene. We confirmed the structure of the reaction product (taxa-4(20),11(12)-dien-5 $\alpha$ -ol) by synthesizing this compound. The responsible biological catalyst was characterized as a cytochrome P450 (heme thiolate protein). *In vivo* studies confirmed that taxadienol is a biosynthetic intermediate and indicated that the hydroxylation step that produces this product is slow relative to subsequent metabolic transformations.

**Conclusions:** The structure of the first oxygenated intermediate on the taxol pathway establishes that the hydroxylation reaction proceeds with an unusual double bond migration that limits the mechanistic possibilities for subsequent elaboration of the oxetane moiety of taxol. The reaction is catalyzed by a cytochrome P450, suggesting that the seven remaining oxygenation steps in taxol biosynthesis may involve similar catalysts. Because the first oxygenation step is slow relative to subsequent metabolic transformations, it may be possible to speed taxol biosynthesis by isolating and manipulating the gene for the taxadiene-5-hydroxylase that catalyzes this reaction.

Addresses: <sup>1</sup>Institute of Biological Chemistry and Department of Genetics and Cell Biology, Washington State University, Pullman, WA 99164-6340, USA, <sup>2</sup>Department of Chemistry, Colorado State University, Fort Collins, CO 80523, USA and <sup>3</sup>USDA-ARS, Plant, Soil and Nutrition Laboratory, Ithaca, NY 14853-9201, USA.

Correspondence: R Croteau or RM Williams  
e-mail: [croteau@mail.wsu.edu](mailto:croteau@mail.wsu.edu)  
[robert\\_williams@mail.chm.colostate.edu](mailto:robert_williams@mail.chm.colostate.edu)

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## Introduction

The complex diterpenoid taxol (paclitaxel) [1] is a potent antimitotic agent with excellent activity against a range of cancers including ovarian and breast cancer [2,3]. The very limited supply of the drug from the original source, the bark of the Pacific yew (*Taxus brevifolia* Nutt.; Taxaceae), prompted the development of alternative means of production [4,5], such as semisynthesis of taxol and its analog taxotere (docetaxel) [6] from advanced taxane diterpenoid (taxoid) metabolites that are more readily available from natural sources [7,8]. Total synthesis of taxol has also been achieved by several elegant routes [9–12], and references therein, but the yields are too low to be commercially viable. With increasing applications in chemotherapy, both in treatment of additional cancer types and in earlier interventions, the availability and cost of these drugs will remain important issues [13]. It is clear that in the foreseeable future the supply of taxol and its synthetically useful progenitors must continue to rely on

biological methods of production, either in *Taxus* species or in cultures of cells derived from these plants [13]. Improving the biological production yields depends upon a detailed understanding of the biosynthetic pathway, the enzymes catalyzing the sequence of reactions, especially the slow steps, and the genes encoding these proteins.

The biosynthesis of taxol (compound 4, Fig. 1) involves the initial cyclization of geranylgeranyl diphosphate (compound 1), the universal precursor of diterpenoids [14], to taxa-4(5),11(12)-diene (compound 2), followed by extensive oxidative modification of the olefin and elaboration of side chains [15] (Fig. 1). The enzyme that catalyzes this reaction, taxadiene synthase, has been purified from yew stem and characterized [16], and the corresponding cDNA has been recently isolated [17]. The cyclization mechanism involves an intramolecular hydrogen transfer and proceeds without free intermediates [18]. It was unexpected to find that the first committed step of the pathway was cyclization to

taxa-4(5),11(12)-diene (compound **2**), since the olefinic precursor of taxol was traditionally considered to be the taxa-4(20),11(12)-diene isomer. This theory had been based on the observation that a large number of naturally occurring taxoids bear double bonds in these positions, and that the sequential conversion of the 4(20)-exocyclic methylene group to the oxirane and then to the oxetane ring of taxol could be readily imagined [19–21].

Given the efficient incorporation of taxa-4(5),11(12)-diene into taxol and related compounds, and the elimination of the exocyclic double bond isomer as an intermediate [15,16,18], several additional observations are relevant in considering subsequent metabolic transformations of the precursor olefin. No oxygenated taxoids bearing a 4(5) double bond are known, whereas taxoids with the 4(20)-ene-5-oxy functional grouping are exceedingly common [22]. Furthermore, based on the relative abundances of the various classes of oxygenated taxane metabolites, hydroxylation at C5 of the taxane skeleton is thought to be an early step in the pathway [21,23]. These observations suggest that hydroxylation of taxa-4(5),11(12)-diene at C5, with migration of the double bond, is probably the next step in the taxol biosynthetic sequence. Here, we describe the isolation and characterization of a cytochrome P450 hydroxylase from *Taxus* stem and cultured cells that catalyzes the conversion of taxa-4(5),11(12)-diene (compound **2**) to taxa-4(20),11(12)-dien-5 $\alpha$ -ol (compound **3**) (Fig. 1). We also provide evidence that this taxadienol is the second specific intermediate on the pathway to taxol and related taxoids, and that the hydroxylation step is relatively slow compared to subsequent metabolic transformations in the sequence.

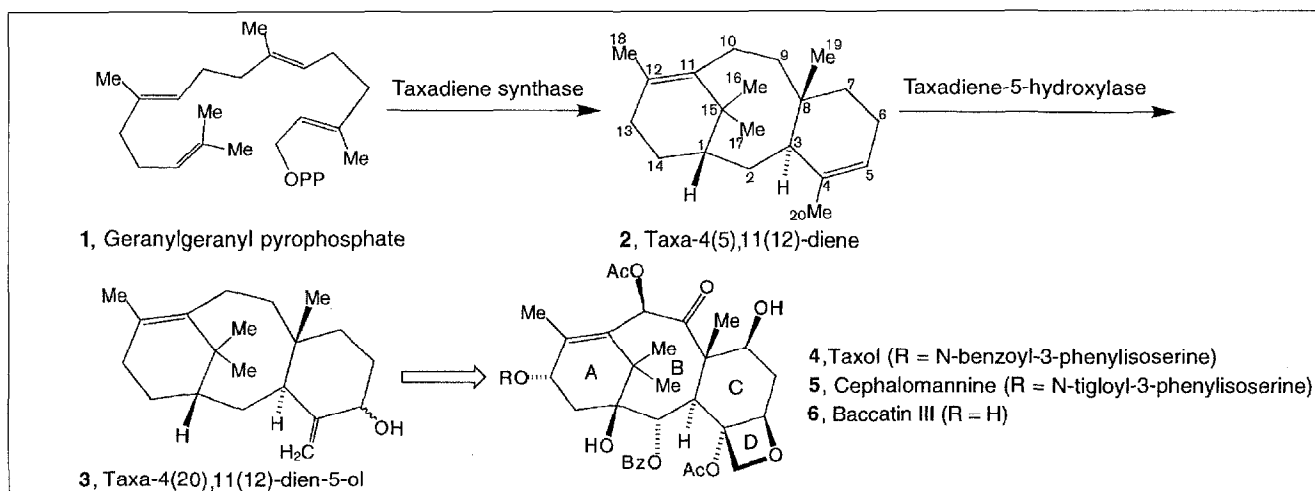
## Results and discussion

### Demonstration of taxadiene oxygenase activity

Since many terpenoid oxygenation reactions are catalyzed by membranous P450 cytochromes [24–26], microsomal preparations from extracts of *T. brevifolia* stems or *T. cuspidata* suspension-cultured cells were examined for their ability to transform [ $^3\text{H}$ ]taxa-4(5),11(12)-diene to more polar products. The microsomal preparations catalyzed the aerobic, NADPH-dependent conversion of the olefin to a product (in 4–14 % yield depending on the tissue) with chromatographic properties similar to that of the diterpenol standard abieta-7(8),13(14)-dien-18-ol on silica gel thin layer chromatography (TLC) and AgNO<sub>3</sub> argentation TLC. Gas-chromatography mass spectrometry (GC–MS) analysis (selected ion monitoring (SIM) mode) of the isolated product indicated that a metabolite (retention time ( $R_t$ )  $13.39 \pm 0.01$  min) with ions at  $m/z$  281, 273, 270 and 255 (ratio ~4:2:1:4) was present, consistent with the P<sup>+</sup>, P<sup>+</sup>-15(CH<sub>3</sub>), P<sup>+</sup>-18(H<sub>2</sub>O), and P<sup>+</sup>-15-18 ion respectively, of a diterpene alcohol. Formation of the putative diterpenol from taxadiene was not supported by cumene hydroperoxide or H<sub>2</sub>O<sub>2</sub>, or by the combination of Fe<sup>2+</sup>, ascorbate and  $\alpha$ -ketoglutarate, eliminating these alternative modes of olefin hydroxylation.

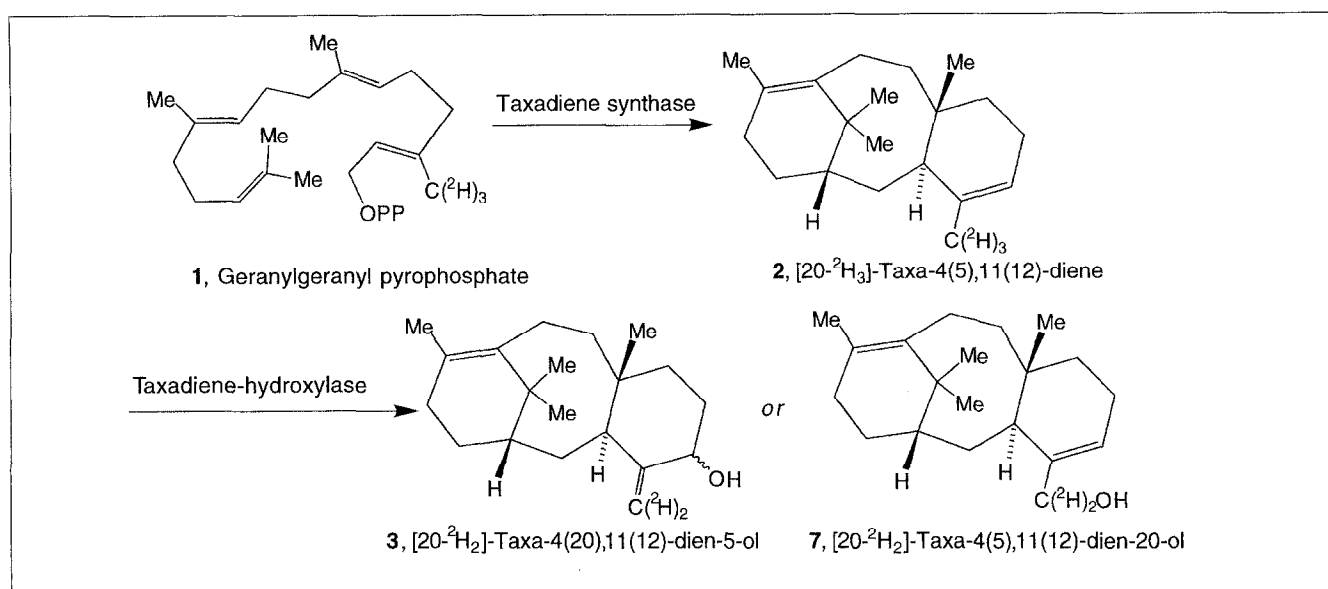
In a preliminary experiment to define the presumptive diterpenol product, [ $^{20-2}\text{H}_3$ ]taxa-4(5),11(12)-diene was prepared biosynthetically from [ $^{20-2}\text{H}_3$ ]geranylgeranyl diphosphate using the partially purified taxadiene synthase from *T. brevifolia* [16,18]. The chromatographically purified olefin (~80 nmol) was then used as a substrate in the oxygenase reaction and the product was isolated by TLC as before. GC–MS analysis (SIM mode) of the

Figure 1



Cyclization of geranylgeranyl diphosphate (compound **1**) to taxa-4(5),11(12)-diene (compound **2**) and elaboration of the olefin to taxol (compound **4**), cephalomannine (compound **5**), or baccatin III

(compound **6**) via a proposed taxa-4(20),11(12)-dien-5-ol intermediate (compound **3**). OPP denotes the diphosphate moiety, Bz the benzoyl group and Ac the acetyl group.

**Figure 2**

Preparation of [20-<sup>2</sup>H<sub>3</sub>]taxa-4(5),11(12)-diene (compound 20-<sup>2</sup>H<sub>3</sub>-2) from [20-<sup>2</sup>H<sub>3</sub>]geranylgeranyl diphosphate (compound 20-<sup>2</sup>H<sub>3</sub>-1) using taxadiene synthase, and conversion of the olefin by the microsomal

oxygenase to a diterpenol with the loss of one deuterium atom. The most likely products are taxa-4(20),11(12)-dien-5-ol (compound 3) or taxa-4(5),11(12)-dien-20-ol (compound 7).

purified material, in this instance, revealed the presence of ions at  $m/z$  290 ( $P^+$ ), 275 ( $P^+-15$ ), 272 ( $P^+-18$ ) and 257 ( $P^+-33$ ). The shift upwards of these diagnostic ions by 2 amu (not 3) indicates the loss of 1 deuterium atom from C20 in the transformation, which can be accounted for by the conversion of taxa-4(5),11(12)-diene (compound 2) to taxa-4(20),11(12)-dien-5-ol (compound 3) or to taxa-4(5),11(12)-dien-20-ol (compound 7) (Fig. 2). Since taxoids bearing a C20 hydroxyl group have not been reported amongst the >100 naturally occurring taxane metabolites now known [22], the result of oxygenation of the deuterated substrate suggested that taxa-4(5),11(12)-diene was hydroxylated at C5 with concomitant allylic migration of the C4,C5 double bond.

#### Syntheses of taxa-4(5),11(12)-dien-5 $\alpha$ -ol and 5 $\beta$ -ol

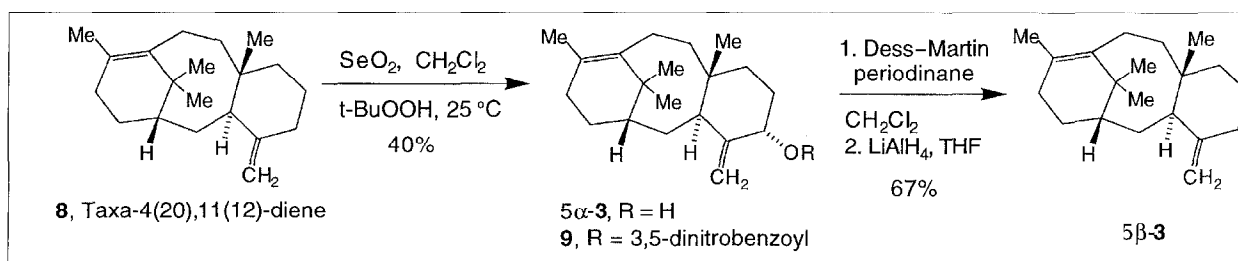
To obtain the 5 $\alpha$ - and 5 $\beta$ -epimers of taxa-4(5),11(12)-dien-5-ol (compound 3) for comparative purposes, we used our previously reported total synthesis of taxa-4(5),11(12)-diene (compound 2) and taxa-4(20),11(12)-diene (compound 8) [27]. Taxa-4(20),11(12)-diene (compound 8) was oxidized with  $\text{SeO}_2$ - $t\text{BuOOH}$  to give a single hydroxylation product in 40 % yield (compound 5 $\alpha$ -3, Fig. 3). Extensive NMR-based structural investigations of this substance (<sup>1</sup>H NMR, nuclear Overhauser enhancement spectroscopy (NOESY), double quantum filtered correlation spectroscopy (DQF-COSY), heteronuclear multiple quantum coherence (HMQC) spectroscopy and heteronuclear multiple bond correlation (HMBC) spectroscopy, see Supplementary material) revealed that this

product was exclusively the 5 $\alpha$ -stereoisomer. The corresponding 5 $\beta$ -stereoisomer (compound 5 $\beta$ -3) was prepared from compound 5 $\alpha$ -3 by oxidation to taxa-4(20),11(12)-dien-5-one followed by stereoselective reduction with  $\text{LiAlH}_4$ . The corresponding, highly crystalline 3,5-dinitrobenzoate (compound 9) of compound 5 $\alpha$ -3 was also prepared for co-crystallization studies.

#### Identification of the biosynthetic product

Chromatographic comparison of the 5 $\alpha$ - and 5 $\beta$ -epimers of taxa-4(20),11(12)-dien-5-ol (compound 3, Fig. 2) to the biosynthetic product prepared from synthetic ( $\pm$ )-[20-<sup>3</sup>H]taxadiene with *Taxus* microsomes showed that the labeled diterpenol has identical chromatographic properties to the authentic 5 $\alpha$ -alcohol (compound 5 $\alpha$ -3) on silica gel TLC (retention factor ( $R_f$ ) 0.5 with pentane:ether, 3:1 v/v) and  $\text{AgNO}_3$ -argentation TLC ( $R_f$  0.5 with pentane:ether, 3:2 v/v). The  $\beta$ -stereoisomer (compound 5 $\beta$ -3) differs substantially in chromatographic behavior on both silica gel TLC ( $R_f$  0.3 with pentane:ether, 3:1 v/v) and argentation TLC ( $R_f$  0.4 with pentane:ether, 3:2 v/v), eliminating this diastereomer as the possible biosynthetic product. Preparative incubations of [<sup>3</sup>H]taxadiene with *Taxus* microsomes were carried out to accumulate sufficient biosynthetic product (~3 nmol) for full spectrum GC-MS analysis. Comparison of retention time and mass spectrum of the TLC-purified biosynthetic product to those of authentic taxa-4(20),11(12)-dien-5 $\alpha$ -ol (compound 5 $\alpha$ -3) indicated that the two compounds were identical (Fig. 4); the retention time (15.21 min) and mass

Figure 3



Outline of the synthesis of the  $5\alpha$ - and  $5\beta$ -epimers of taxa-4(20),11(12)-dien-5-ol (compounds  $5\alpha$ - and  $5\beta$ -3) from taxa-4(20),11(12)-diene (compound **8**). Compound **9** was prepared for co-crystallization studies.

spectrum of compound  $5\beta$ -3 are readily distinguishable from those of compound  $5\alpha$ -3. Finally, the purified, tritium-labeled biosynthetic product (42.8 nCi) was converted to the 3,5-dinitrobenzoate ester (90 % yield), diluted with authentic ( $\pm$ )-taxa-4(20),11(12)-dien-5 $\alpha$ -yl dinitrobenzoate (compound **9**, 8.0  $\mu\text{mol}$ ), and recrystallized to constant specific activity and melting point ( $4.67 \pm 0.09$  nCi  $\mu\text{mol}^{-1}$ , m.p.  $171^\circ\text{C}$ , decomposing at melting temperature (dec.)). The results of these analyses, including quantitative evaluation of the radiochemical crystallization studies, indicate that a minimum of 87 % of the oxygenation product of taxa-4(5),11(12)-diene (compound **2**) is taxa-4(20),11(12)-dien-5 $\alpha$ -ol (compound  $5\alpha$ -3).

Radiochemically-guided fractionation of a *T. brevifolia* bark extract (0.25  $\mu\text{Ci}$  of ( $\pm$ )-[20- $^3\text{H}$ ]taxa-4(20),11(12)-dien-5 $\alpha$ -ol diluted into an extract of 250 kg dry bark), carried out using silica gel, argentation, and reversed-phase chromatography, before and after alkaline hydrolysis of half of the extract, followed by GC-MS analysis of the partially purified product, demonstrated that the alcohol is present in the 5–10  $\mu\text{g kg}^{-1}$  range and the ester(s) in the 25–50  $\mu\text{g kg}^{-1}$  range. The low abundance of these oxygenated metabolites in bark tissue is in the range of that previously observed for the olefin substrate of the hydroxylase (5–10  $\mu\text{g taxadiene kg}^{-1}$ ). Thus, like the enzymatic cyclization to taxadiene [15,16], the oxygenation of taxadiene appears to be a slow metabolic step relative to subsequent transformations in stem tissue since the reaction product does not appreciably accumulate.

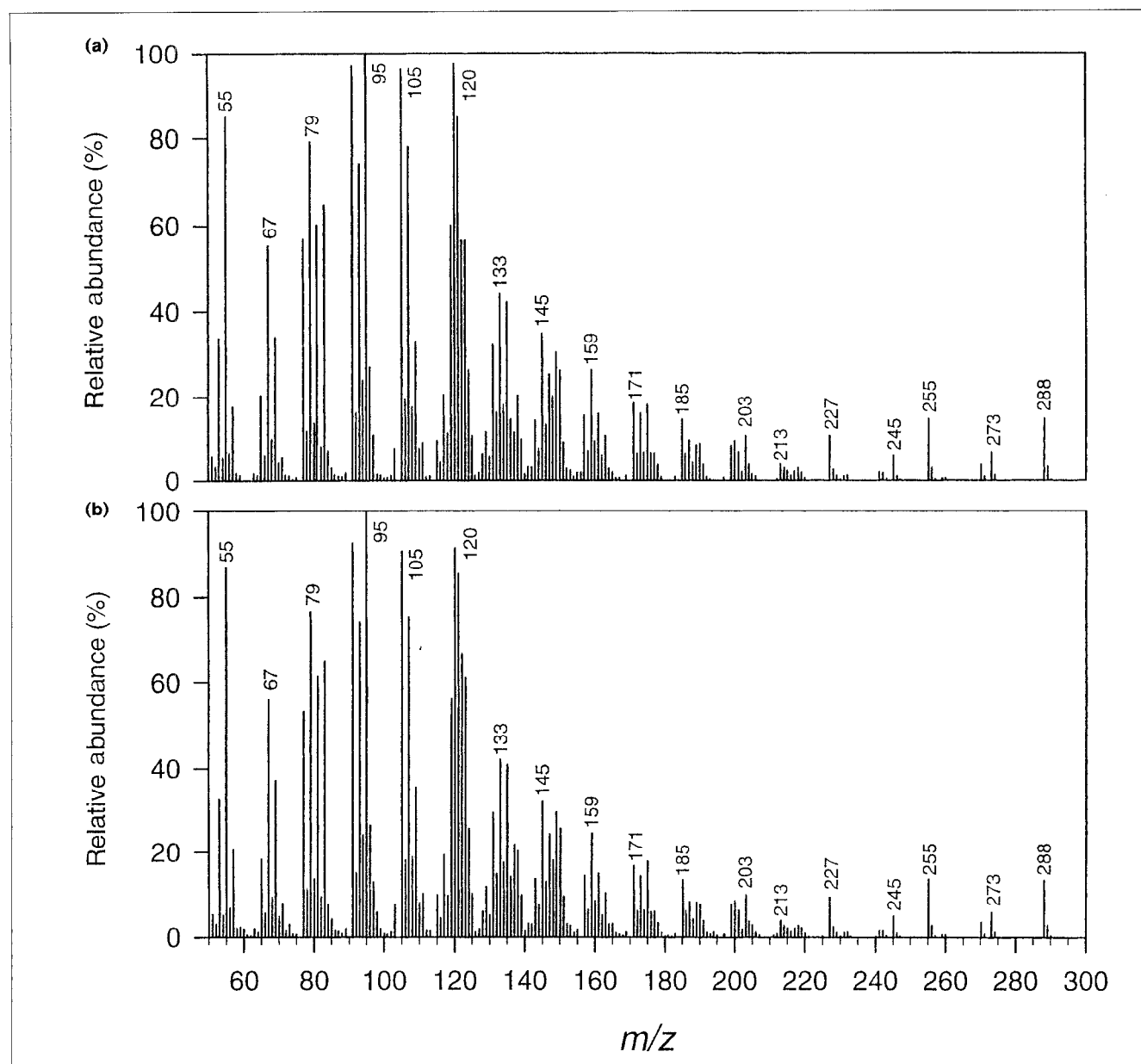
#### Taxa-4(20),11(12)-dien-5 $\alpha$ -ol as a precursor of taxol

To determine if taxa-4(20),11(12)-dien-5 $\alpha$ -ol (compound  $5\alpha$ -3) could serve as a precursor of taxol (compound **4**) and related taxoids, 4.0  $\mu\text{Ci}$  (1  $\mu\text{mol}$ ) of the synthetic tritium-labeled alcohol was suspended in 4.5 ml of buffer and equal portions were vacuum-infiltrated into three batches (1.2 g each) of *T. brevifolia* stem discs. Following incubation for two, four and six days, the labeled products were extracted, and the advanced taxoids, 10-deacetylba-

ccatins (compound 10-deacetyl-6), cephalomannine (compound **5**), and taxol (compound **4**) (see Fig. 1), were isolated by TLC, diluted with 200 mg of the corresponding carrier, and crystallized to constant specific activity. The incorporation levels for the two, four and six day time points were 0.96, 3.07 % for 10-deacetylba-

#### Characterization of the oxygenase

Centrifugal fractionation of tissue extracts from *T. brevifolia* sapling stems and *T. cuspidata* suspension cultured cells indicated that the major portion of oxygenase activity (70 %) resides in the light membrane fraction (190,000g pellet). Approximately 25 % of taxadiene-5-hydroxylase activity was located in the dense membrane fraction (20,000g pellet), probably the result of occlusion of microsomes by dense membranes, with the residual activity (~5 %) associated with the soluble enzyme fraction (190,000g supernatant). The inclusion of phospholipase inhibitors, 10 mM each of procaine and leupeptin in the isolation buffer doubled the microsomal oxygenase activity, presumably by preserving membrane integrity. The pH optimum for the reaction (in HEPES buffer) was near 7.2, with a ~50% reduction in activity at pH 6.2 or pH 8.2. The taxadiene-5-hydroxylase was completely dependent on NADPH (1 mM), and the inclusion of an NADPH-regenerating system significantly increased activity, further supporting the hypothesis that this reduced pyridine nucleotide cofactor is required for activity (Table 1). The inclusion of 10  $\mu\text{M}$  FMN (saturation at 2.5  $\mu\text{M}$ ) in the reaction mix-

**Figure 4**

GC-MS analysis of the product of the microsomal oxygenase shows that it is identical to synthetic compound 5 $\alpha$ -3. The mass fragmentation patterns are illustrated for (a) authentic ( $\pm$ )-taxa-4(20),11(12)-dien-5 $\alpha$ -ol

( $R_t$  = 13.38 min) and for (b) the diterpenol ( $R_t$  = 13.39 min) obtained from taxa-4(5),11(12)-diene when incubated with *Taxus* microsomes in the presence of NADPH and  $O_2$ .

improved hydroxylase activity (Table 1), presumably by replacing the flavin prosthetic groups lost during microsome isolation from the NADPH-cytochrome P450 reductase required to couple electron transfer from NADPH to the cytochrome in the course of the reaction [28–30]. Under optimum assay conditions, the  $K_M$  value for the substrate ( $\pm$ )-[20- $^3H$ ]taxa-4(5),11(12)-diene was estimated to be  $\sim 6.0 \mu M$  from Lineweaver–Burk plots of the kinetic data. Because the substrate is racemic and the enzyme system is membranous, this value should be

interpreted with caution;  $K_M$  values in the micromolar range are typical of terpenoid hydroxylases [25]. Although the substrate prepared biosynthetically from [1- $^3H$ ]geranylgeranyl diphosphate using the taxadiene synthase ([2- $^3H$ ]taxa-4(5),11(12)-diene) [15,16] was not available in sufficient amounts to permit kinetic studies, the product of the hydroxylation of this presumably enantiomerically pure olefin was identical (by GC-MS) to the product generated from the synthetic, racemic precursor.

Table 1

## Reaction conditions for taxadiene-5-hydroxylase

Conditions	Relative rate
Complete system <sup>a</sup>	100
without flavins	73
without NADPH regenerating system	55
without oxygen	14
without NADPH <sup>b</sup>	0
1:1 CO:O <sub>2</sub> <sup>c</sup>	
dark	85
9:1 CO:O <sub>2</sub> <sup>c</sup>	
dark	18
450 nm light	73

<sup>a</sup>Complete system contains 26  $\mu\text{M}$  taxa-4(5),11(12)-diene, 1 mM NADPH, 5 mM glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 2.5  $\mu\text{M}$  FMN, 2.5  $\mu\text{M}$  FAD and 500  $\mu\text{g}$  microsomal protein per ml.

<sup>b</sup>Relative to boiled control.

<sup>c</sup>Relative to corresponding dark and light incubations (complete system) without CO.

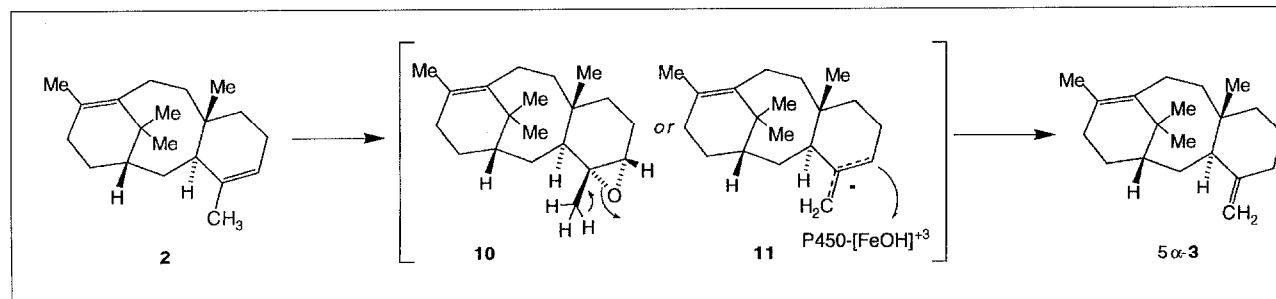
Excluding molecular oxygen from the reaction mixture greatly reduced hydroxylase activity (Table 1); however, the failure to demonstrate an absolute oxygen requirement probably resulted from the inability to remove residual O<sub>2</sub> from the assay buffer, coupled with a relatively high affinity of the cytochrome for this reactant. Hydroxylation of taxadiene was inhibited by CO in the dark, and this inhibition was partially reversed by blue (450 nm) light (Table 1). Finally, the reaction was completely inhibited by clotrimazole and miconazole (at 3  $\mu\text{M}$ ) and was partially inhibited by cytochrome *c* (I<sub>50</sub> ~400  $\mu\text{M}$ ). In all defining characteristics (microsomal location, NADPH and oxygen dependence, blue light reversal of CO inhibition, inhibition by substituted imidazoles and cytochrome *c*) the taxadiene hydroxylase resembles a typical cytochrome P450 mixed function oxidase [25,26,31–33].

## Mechanistic considerations

Based on precedented modes of cytochrome P450 mediated oxidations of olefinic substrates ([34–41] references therein), we considered two possible mechanisms for the oxidation at C5 of taxadiene (compound **2**) which involve a formal allylic transposition (Fig. 5). The first possibility involves preliminary conversion of the double bond of compound **2** to the corresponding epoxide **10**, followed by ring opening and elimination of a proton from the C20 methyl group to yield allylic alcohol **5 $\alpha$ -3**. However, no evidence for such a free epoxide intermediate has been obtained by radio-chromatography or GC–MS analysis of the enzymatic reaction products. Alternatively, cytochrome P450-mediated abstraction of a hydrogen atom from the C20 methyl group of substrate would yield allylic radical **11**, which could undergo oxidation to compound **5 $\alpha$ -3**.

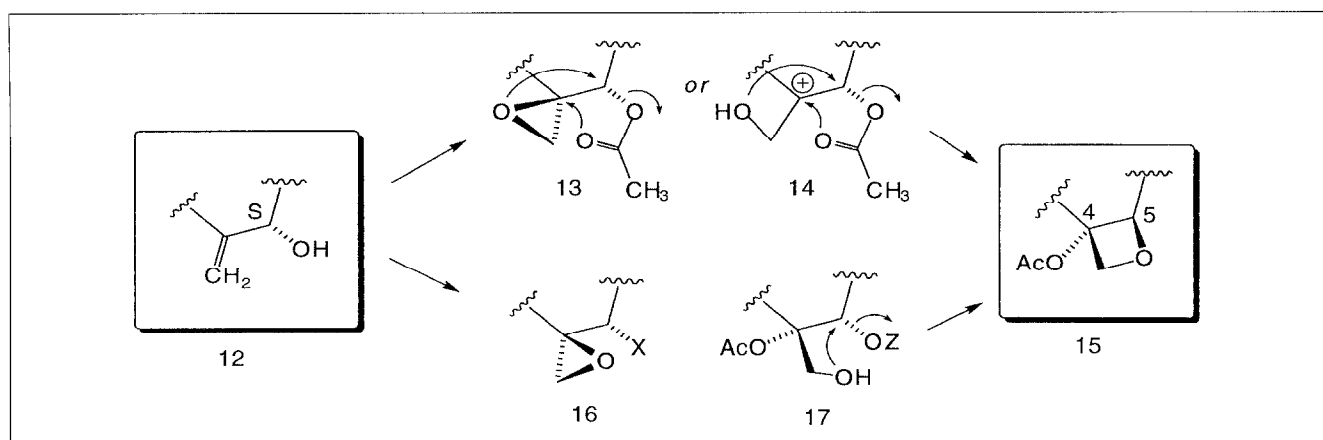
We reasoned that these two mechanistic possibilities might be distinguishable using [20-<sup>2</sup>H<sub>3</sub>]taxadiene substrate, since epoxide ring-opening should not involve a rate-limiting C20–H bond-breaking step, whereas the allylic radical pathway might involve a sufficiently slow C20–H bond-breaking step to be detectable by the presence of the primary deuterium kinetic isotope effect on the overall reaction rate. To examine this possibility, we synthesized [20-<sup>2</sup>H<sub>3</sub>]taxa-4(5),11(12)-diene (see Supplementary material) and tested it as a substrate with microsomes, using parallel incubations with unlabeled compound **2** as a control. Product quantification by using abietadienol as an internal standard, unexpectedly revealed that the deuterated substrate yielded slightly more taxa-4(20),11(12)-dien-5 $\alpha$ -ol than did the unlabeled substrate. This result indicates that, within the limits of experimental error, there was no observable deuterium kinetic isotope effect on the overall reaction rate. Although this lack of rate suppression might argue in favor of the epoxidation/ring-opening mechanism, it is possible that the rate of substrate binding or product release is rate-limiting.

Figure 5



Two possible mechanisms for the taxadiene-5-hydroxylase-catalyzed conversion of taxadiene **2** to taxadienol **5 $\alpha$ -3** based on precedented P450-mediated olefin oxidations involving an allylic transposition. The first mechanism proceeds by preliminary epoxidation of the 4(5) double

bond (to form compound **10**) followed by ring opening and deprotonation at C20; the second exploits hydrogen abstraction at C20, rearrangement of the resulting allylic radical (compound **11**) and oxygen rebound at C5.

**Figure 6**

Possible mechanisms for the construction of the oxetane ring (compound **15**) from the 4(20)-ene-5 $\alpha$ -hydroxy functional grouping (compound **12**). Oxidation of the the 4(20) double bond forms an epoxide ring that may be acetylated to form compound **13**.

Intramolecular acetate migration and epoxide-ring opening could then furnish the oxetane moiety. Alternatively, the epoxide structure in compound **16** may be subjected to intermolecular acetate opening that could promote oxetane ring closure via compound **17**.

release by the cytochrome, or that of some other step of the reaction cycle, may be slow enough to mask a kinetic isotope effect on the hydrogen abstraction; there is precedent for such masking in cytochrome P450-catalyzed oxidations [38]. We are currently working on preparing the CHD<sub>2</sub>-analog of the substrate, to examine the intramolecular isotope effect on deprotonation at C20, and synthesizing epoxide **10** to determine the metabolic relevance of this hypothetical intermediate.

The observation that the taxadiene-5-hydroxylase affords exclusively 5 $\alpha$ (*S*)-stereochemistry is consistent with the natural occurrence of a large number of taxoids bearing this configuration [22], and suggests several reasonable mechanisms for the elaboration of the oxetane (D) ring of taxol and related metabolites [20,21] (Fig. 6). Oxidation of the 4(20) double bond of compound **12** to the corresponding epoxide followed by acylation would provide compound **13**. Intramolecular acetate migration concomitant with epoxide ring-opening or a closely related step-wise migration (as in compound **14**) could then furnish the oxetane moiety (compound **15**). Alternatively, conversion of the 4(20) double bond to an activated epoxide (compound **16**) followed by intermolecular acetate opening of the epoxide could promote oxetane ring closure via compound **17**. Efforts are currently under way to exploit double-labeling experiments to examine the timing and mechanism of elaboration of the D ring.

### Significance

Chemists and biologists alike have been drawn to the study of taxol because of its spectrum of antineoplastic activities [2,3], its unique mechanism of action in arresting the cell cycle by stabilization of microtubule

dynamics [42,43], and the synthetic and biosynthetic challenges posed by the complex and densely functionalized diterpenoid ring system. The limited supply of taxol has hindered the development and use of this natural product as a chemotherapeutic agent since its discovery over 25 years ago in the bark of the Pacific yew [1]. Intensive efforts have thus been made to develop alternative means for the commercial production of this drug. To date, totally synthetic methods have not proven successful as a means of commercial production due to the highly complex nature of the target, which mandates lengthy, expensive synthetic routes with low overall yields [44]. As an interim measure, semi-synthetic approaches have been devised using advanced taxoid metabolites, such as 10-deacetyl-baccatin III, which can be obtained from renewable sources, such as needles of the European yew (*T. baccata*) [45]. From the pharmaceutical manufacturing point of view, efficient cell culture production methods would be most desirable. Taxol is produced in *Taxus* cell cultures [46], but production yields are such that this approach is not yet commercially viable.

Improvement of biologically-based processes should be based on a thorough understanding of the target pathway and its rate limiting steps. To this end, we have undertaken a systematic, stepwise approach to deciphering the biosynthesis of taxol and identifying the slow steps in this complex, extended reaction sequence. The first dedicated step of the pathway, at which primary metabolism is diverted to the production of taxol, is the cyclization of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene, establishing the taxane skeleton for subsequent oxidative elaboration [15,16]. This taxadiene

synthase-catalyzed step is very slow and is thus an important target for manipulation; the cDNA encoding this synthase has recently been isolated [17].

Here, we have focused on the first oxygenation of taxadiene, a step which studies *in vivo* indicate may also limit pathway flux. The identification of taxa-4(20),11(12)-dien-5 $\alpha$ -ol as the second specific intermediate *en route* to taxol has important mechanistic implications for the hydroxylation reaction, which proceeds with a formal allylic rearrangement, and it also constrains the possible means for construction of the oxetane ring of taxol. Identification of the responsible enzyme as a P450 cytochrome, and the development of a specific and sensitive assay, permit a homology-based approach to cloning the corresponding gene. *Agrobacterium tumefaciens*-mediated transformation of *Taxus* species has been described, and the resulting callus cells were shown to produce taxol [47]. It should soon be possible to engineer cells that overexpress the enzymes catalyzing the first two, very slow steps of the pathway to promote improved production yields in these systems.

## Materials and methods

### Experimental materials

The sources of *T. brevifolia* saplings (2–4 years old) and *T. cuspidata* suspension-cultured cells (14 days post-transfer) have been described [15,48], as have the preparations of [1-<sup>3</sup>H]geranylgeranyl diphosphate at 90 Ci mol<sup>-1</sup> [49] and [20-<sup>2</sup>H<sub>3</sub>]geranylgeranyl diphosphate at >95 atom % <sup>2</sup>H [18], and the source of abietadienol [50] and of the taxoid standards 10-deacetylbaicatin III, cephalomannine and taxol [15]. The total synthesis of taxa-4(5),11(12)-diene and taxa-4(20),11(12)-diene has been described [27]. Modification of this strategy afforded [20-<sup>3</sup>H<sub>3</sub>]-labeled (16 Ci mol<sup>-1</sup>) and [20-<sup>2</sup>H<sub>3</sub>]-labeled (99 atom % <sup>2</sup>H) taxa-4(5),11(12)-diene (see Supplementary material).

### Syntheses

#### Taxa-4(20),11(12)-dien-5 $\alpha$ -ol (compound 5 $\alpha$ -3)

To a 0.04 M solution of SeO<sub>2</sub> (7 mg, 0.07 mmol) in CH<sub>2</sub>Cl<sub>2</sub> was added *t*BuOOH (26 ml, 0.26 mmol) and the resulting mixture was allowed to stir for 0.5 h. To this solution was added a 0.07 M solution of (+)-taxa-4(20),11(12)-diene (compound 8) (36 mg, 0.13 mmol) in CH<sub>2</sub>Cl<sub>2</sub> and the mixture was allowed to stir for 8 h. The reaction mixture was concentrated and the crude oil was purified by preparative TLC (silica gel, hexanes:ethyl acetate, 4:1 v/v) to give 14 mg (39%) (*R*<sub>f</sub> = 0.75, hexanes:ethyl acetate, 2:1 v/v) of product (compound 5 $\alpha$ -3) as a colorless oil. IR (neat) 3397, 2925, 1448 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 500 MHz)  $\delta$  4.91 (dd, *J* = 1.4, 1.4 Hz, 1H), 4.62 (ddd, *J* = 1.7, 1.1, 0.5 Hz, 1H), 4.23 (dd, *J* = 2.7, 2.7 Hz, 1H), 3.31–3.28 (m, 1H), 2.82 (dt, *J* = 13.5, 5.3 Hz, 1H), 2.36–2.28 (m, 1H), 2.24 (ddd, *J* = 13.1, 13.1, 6.8 Hz, 1H), 2.1–1.93 (m, 3H), 1.87 (ddd, *J* = 18.6, 10.6, 3.1 Hz, 1H), 1.81 (dd, *J* = 1.1, 1.1 Hz, 3H), 1.78–1.68 (m, 3H), 1.62 (ddd, *J* = 15.5, 5.9, 2.4 Hz, 1H), 1.54 (ddd, *J* = 15.3, 5.3, 2.2 Hz, 1H), 1.32 (s, 3H), 1.28–1.18 (m, 2H), 1.17 (s, OH, 1H), 1.02 (s, 3H), 0.97 (ddd, *J* = 13.3, 4.3, 2.9 Hz, 1H), 0.59 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz)  $\delta$  155.9, 136.8, 130.5, 108.7, 74.6, 43.5, 40.0, 39.9, 39.2, 35.4, 32.7, 30.8, 30.16, 30.13, 28.0, 25.4, 24.7, 22.9, 22.1, 21.3. High-resolution mass spectroscopy (HRMS) calc'd for C<sub>20</sub>H<sub>32</sub>O: 288.2453, found: 288.2448. The synthesis of [20-<sup>3</sup>H<sub>2</sub>]taxa-4(20),11(12)-dien-5 $\alpha$ -ol (4.0 Ci mol<sup>-1</sup>) is described in the Supplementary material.

**Taxa-4(20),11(12)-dien-5 $\alpha$ -3,5-dinitrobenzoate (9)**  
To a 0.03 M solution of taxa-4(20),11(12)-dien-5 $\alpha$ -ol (compound 5 $\alpha$ -3) (20 mg, 0.07 mmol) in CH<sub>2</sub>Cl<sub>2</sub> was added pyridine (1 mmol) and the resulting mixture was cooled to 0 °C. To this mixture was added 3,5-dinitrobenzoyl chloride (74 mg, 0.32 mmol) and the mixture was allowed to stir for 4 h as it warmed to 25 °C. The mixture was quenched with saturated aqueous NaHCO<sub>3</sub> and extracted with ether (3 x 10 ml). The organic layer was washed with brine (20 ml), dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The residue was purified by preparative TLC (silica gel, hexanes:ethyl acetate, 4:1 v/v) to give 26 mg (79%) (*R*<sub>f</sub> = 0.28, hexanes:ethyl acetate, 4:1 v/v) of product (compound 9) as a yellow solid. An analytical sample was purified by recrystallization (m.p. 101 °C). IR (neat) 3098, 2923, 1723, 1626, 1544, 1451, 1344 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz)  $\delta$  9.22 (t, *J* = 2.1 Hz, 1H), 8.12 (d, *J* = 2.1 Hz, 2H), 5.58 (t, *J* = 3 Hz, 1H), 5.28 (s, 1H), 4.98 (dd, *J* = 4.2 Hz, 1H), 2.77 (apparent dt, *J* = 7.2, 14.7 Hz, 1H), 2.51–2.44 (m, 2H), 2.35–1.95 (m, 5H), 1.85–1.75 (m, 3H), 1.48–1.37 (m, 2H), 1.35 (s, 3H), 1.28–1.15 (m, 3H), 0.7 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz)  $\delta$  155.9, 136.8, 130.5, 108.7, 74.6, 43.5, 40.0, 39.9, 39.2, 35.4, 32.7, 30.8, 30.16, 30.13, 28.0, 25.4, 24.7, 22.9, 22.1, 21.3. HRMS calc'd for C<sub>27</sub>H<sub>34</sub>N<sub>2</sub>O<sub>6</sub>: 482.2417, found: 482.2417.

For use with the labeled biosynthetic sample, the reaction was modified to the 0.5  $\mu$ mol scale (42.8 nCi of product per  $\mu$ mol of taxadienol as carrier) with the reagent in ten-fold excess above, gave the product in 90% isolated yield. The product was diluted with additional carrier (7.6  $\mu$ mol of the dinitrobenzoate) and repeatedly crystallized from 30% aqueous methanol.

#### 5-Keto-taxa-4(20),11(12)-diene

To a 0.01 M solution of taxa-4(20),11(12)-dien-5 $\alpha$ -ol (compound 5 $\alpha$ -3) (6 mg, 0.02 mmol) in CH<sub>2</sub>Cl<sub>2</sub> was added Dess–Martin reagent (29 mg, 0.07 mmol); the resulting mixture was stirred for 2 h. Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O (15 mg, 0.06 mmol) was added and the solution of NaHCO<sub>3</sub> (4 mg, 0.046 mmol); this solution, in turn, was then added to the reaction mixture which was stirred for 30 min. Saturated aqueous NaHCO<sub>3</sub> solution (10 ml) was added and the mixture was extracted with ether (3 x 10 ml). The organic layer was washed twice with saturated aqueous NaHCO<sub>3</sub> solution, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The residue was purified by preparative TLC (silica gel, hexanes:ethyl acetate, 4:1 v/v) to give 4 mg (67%) (*R*<sub>f</sub> = 0.57; hexanes:ethyl acetate, 4:1 v/v) of product ketone as a colorless oil. IR (neat) 2928, 1697, 1617 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz)  $\delta$  5.81 (d, *J* = 2.5 Hz, 1H), 5.58 (dd, *J* = 2.5 Hz, 1H), 2.87 (sextet, *J* = 2.5 Hz, 1H), 2.75 (apparent dq, *J* = 2.5 Hz, 1H), 2.51–2.44 (m, 2H), 2.35–1.95 (m, 5H), 1.85–1.75 (m, 3H), 1.48–1.37 (m, 2H), 1.35 (s, 3H), 1.28–1.15 (m, 3H), 0.7 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz)  $\delta$  203.9, 129.8, 116.6, 43.2, 40.4, 39.6, 39.3, 37.9, 36.5, 33.5, 27.9, 25.5, 24.5, 23.5, 22.4, 21.5. HRMS calc'd for C<sub>20</sub>H<sub>30</sub>: 286.2375, found: 286.2378.

#### Taxa-4(20),11(12)-dien-5 $\beta$ -ol (compound 5 $\beta$ -3)

To a 0.01 M solution of 5-keto-taxa-4(20),11(12)-diene (described above) (4 mg, 0.014 mmol) in tetrahydrofuran was added LiAlH<sub>4</sub> (0.6 mg, 0.014 mmol) and the resulting mixture was allowed to stir for 15 min. The reaction mixture was quenched with H<sub>2</sub>O (10 ml) and the aqueous layer was extracted with ether (2 x 10 ml). The organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated to give 4 mg (100%) of the product (compound 5 $\beta$ -3) as a colorless oil. An analytical sample was purified by preparative TLC (silica gel, hexanes:ethyl acetate, 4:1 v/v; *R*<sub>f</sub> = 0.25). IR 2927, 2858, 1646, 1452 cm<sup>-1</sup>. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 300 MHz)  $\delta$  4.68 (s, 1H), 3.92–3.8 (m, 1H), 2.83–2.69 (m, 1H), 2.4–2.2 (m, 1H), 2.15–1.82 (m, 5H), 1.82–1.5 (m, 3H), 1.5–1.1 (m, 4H), 1.3 (s, 3H), 1.0 (s, 3H), 0.6 (s, 3H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 75 MHz)  $\delta$  155.7, 137.7, 129.6, 102.2, 74.2, 43.5, 40.0, 39.9, 39.2, 35.4, 32.7, 30.8, 30.16, 30.13, 28.0, 25.4, 24.7, 22.9, 22.1, 21.3.



39.5, 39.3 35.5, 33.3, 30.8, 30.1, 29.3, 25.4, 24.7, 22.9, 22.7, 21.9. HRMS calc'd for  $C_{20}H_{32}O$ : 288.2453, found: 288.2449.

#### Enzyme preparation and assay

Sapling stem sections (60 g) were frozen in liquid  $N_2$ , the needles removed, and the bark and cambium pulverized in a hammer mill [49,51]. The resulting frozen powder was added to HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.6, 5 ml  $g^{-1}$  fresh tissue wt) containing 1 mM  $MgCl_2$ , 2 mM EGTA, 1 mM 4'-bromoacetophenone, 10 mM procaine-HCl, 10 mM lidocaine-HCl, 5 mM sodium ascorbate, 5 mM  $Na_2S_2O_5$ , 5 mM dithiothreitol, 0.1 mM PMSF, 1  $\mu g$  leupeptin  $ml^{-1}$ , 15 % (v/v) glycerol, 1 % (w/v) polyvinylpyrrolidone (relative molecular mass 40,000), 1 % (w/v) polyvinylpolypyrrolidone, and 10 % (w/v) Amberlite XAD-4 polystyrene resin. The mixture was stirred at 4 °C for 30 min then filtered through eight layers of cheesecloth. The filtrate was centrifuged at 3,000g for 20 min to remove cellular debris then again at 20,000g for 20 min to remove dense membranes. This supernatant was then centrifuged at 190,000g for 90 min to obtain the microsomal pellet and the soluble enzyme fraction. The microsomes were resuspended with the aid of a Ten-Broeck homogenizer in 3 ml of assay buffer consisting of 25 mM HEPES (pH 7.2), 1 mM EGTA, 10 mM  $MgCl_2$ , 25 mM KCl, 5 mM dithiothreitol (not included in cytochrome *c* inhibition experiments), 1  $\mu g$  leupeptin  $ml^{-1}$ , and 20 % (v/v) glycerol. Microsome suspensions were aliquoted, frozen in liquid nitrogen and stored at -80 °C for later use; these preparations were stable for at least two weeks. Microsomes from *T. cuspidata* suspension-cultured cells were prepared as above, except that the filtered, frozen cells were homogenized using a chilled mortar and pestle instead of the hammer mill.

Assays were performed in 10 ml screw-capped culture tubes containing 500  $\mu g$  of microsomal protein, 2.5 nmol each of FMN and FAD, 1  $\mu mol$  NADPH, 5  $\mu mol$  glucose-6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase in a total volume of 1 ml of the assay buffer described above. The reactions were initiated by the addition in 5  $\mu l$  hexane of ( $\pm$ )-[20- $^3H$ ]*taxa*-4(5),11(12)-diene (26 nmol, saturation) or [2- $^3H$ ]*taxa*-4(5),11(12)-diene (typically, 1.8 nmol). The latter substrate was prepared biosynthetically from [1- $^3H$ ]geranylgeranyl diphosphate using taxadiene synthase [16,18], and was used in preliminary studies before the synthetic substrate was available. Following incubation for 70 min in the dark at 31 °C with gentle shaking, the reaction mixture was chilled on ice, saturated with NaCl, extracted with 3 x 1 ml of pentane:ether (9:1 v/v) and the extract passed over a short column of  $MgSO_4$  followed by an additional 3 ml wash. The eluate was evaporated to dryness, and the residue was redissolved in 3 ml of pentane that was passed over a 5 mm x 40 mm column of silica gel (Mallinckrodt SilicAR 60A) and washed with 4 ml of pentane to provide the olefin fraction containing residual substrate. The silica gel column was next eluted with pentane:ether (9:1 v/v) to provide the diterpenol fraction, and was finally eluted with ether (this latter fraction contained low, variable amounts of a label product(s) with chromatographic properties consistent with a diterpene diol). An aliquot of the diterpenol fraction was evaluated by liquid scintillation counting to determine conversion rate and, when appropriate, the product was separated by TLC.

A low, but significant, level of background activity was observed in both boiled controls and in controls lacking NADPH. Controls were included in all experiments and the appropriate background corrections made. All assays were conducted under linear conditions with respect to protein concentration and time. Activity levels measured for taxadiene-5-hydroxylase ranged from 0.14 to 1.7 nmol product per hour per mg protein. Microsomes from stems and cultured cells could be used interchangeably; however, the suspension cultures usually provided the higher activity levels per gram of tissue.

#### Enzyme characterization

To examine the oxygen requirement for hydroxylase activity, an oxygen scavenging system (consisting of 10 units glucose oxidase, 6  $\mu mol$

$\beta$ -D-glucose and 1,300 units catalase [52]) was included in the assay, and the reaction mixture was flushed with Ar through the septum cap for 2 min prior to substrate addition. To test inhibition by substituted imidazoles, these compounds were dissolved in dimethylsulfoxide and added (in 5  $\mu l$ ) to the assays 5 min prior to substrate addition (dimethylsulfoxide alone had no effect on taxadiene hydroxylase activity). To evaluate CO inhibition and blue-light reversal, the reaction mixtures were flushed for 2 min with gas mixtures consisting of 10 %  $O_2$  and 90 %  $CO$ , or 10 %  $O_2$ , 10 %  $CO$  and 80 %  $N_2$ , prior to substrate addition. Reactions were incubated in the dark (masked) or were irradiated with blue light (maximum intensity near 450 nm) generated by filtering (0.2 mm filter sheet, Edmund Scientific, Co.) the beam from a 100 W mercury spotlight. All assays were performed in duplicate and typically repeated three times. The data presented are averages with standard error within 10 %.

#### In vivo incorporation experiments

*In vivo* incorporation experiments with *T. brevifolia* sapling stem discs were carried out exactly as described previously [15], except that the 1.5 ml of incubation buffer for each of the 2, 4 and 6-day time points contained 0.22 mM (0.33  $\mu mol$ , 1.33  $\mu Ci$ ) of ( $\pm$ )-[20- $^3H$ ]*taxa*-4(20),11(12)-dien-5 $\alpha$ -ol. The labeled taxoids produced by the tissue were recovered as before; the 10-deacetylbaccatin III, cephalomannine and taxol were isolated by preparative TLC, and each was diluted with 200 mg of authentic carrier and crystallized from aqueous methanol to constant specific activity and melting point as previously described [15]. For 2, 4 and 6-day time points, respectively, repeated crystallization gave 10-deacetylbaccatin III (34.7, 59.6 and 111.8 nCi  $mmol^{-1}$ , m.p. 242–245 °C), cephalomannine (8.8, 15.4 and 19.3 nCi  $mmol^{-1}$ , m.p. 184–186 °C dec.) and taxol (15.9, 33.0 and 43.8 nCi  $mmol^{-1}$ , m.p. 198–203 °C dec.). All melting point values (Fisher-Johns) are in agreement with the literature [22].

#### Analysis of bark extracts

Initial processing of the extract from 750 kg of *T. brevifolia* bark powder has been described, as have the general protocols for radiochemically-guided fractionation of this material [15]. Following isolation of the hydrocarbon fraction by hexane elution through silica gel, two aliquots of the ether eluate (equivalent to 250 kg of starting material) were diluted with 0.25  $\mu Ci$  of [20- $^3H$ ]*taxa*-4(20),11(12)-dien-5 $\alpha$ -ol, one of which was then hydrolyzed with excess 0.1 N KOH to liberate alcohols which were recovered by ether extraction. Subsequent fractionation involved silica gel column chromatography (0–35 % gradient of ether in hexane), argentation column chromatography (10 %  $AgNO_3$ -silica gel with a 0–50 % gradient of ether in hexane) and reversed-phase column chromatography on Davisil (Alltech  $C_{18}$  on silica, 0–20 %  $CCl_4$  in acetonitrile) followed by TLC (silica gel with hexane:ether, 3:1 (v/v)) to provide a fraction ( $R_f \approx 0.5$ ) suitable for GC-MS analysis.

#### Analytical procedures

GC-MS analyses were performed on a Hewlett-Packard 6890 GC-MSD system using a bonded, FSOT column (0.25 mm inner diameter x 30 m) coated with a 0.2  $\mu m$  film of AT1000 (Alltech Associates). Cool, on-column injection was used with a He flow rate of 0.7  $ml min^{-1}$  and a temperature program from 50 °C (2 min hold) to 230 °C at 40 °C  $min^{-1}$ . Spectra (at 70 eV) were recorded in the total ion or selected ion monitoring (SIM) mode. Procedures for NMR spectrometry are described in the Supplementary material.

Analytical TLC was performed on 0.2 mm silica gel sheets (Chromatogram Kodak) using pentane: ether (3:1, v/v) as developing solvent, with visualization using  $I_2$  vapor. Preparative TLC was performed in 1 mm plates (silica gel G or silica gel G containing 8 %  $AgNO_3$ ) using pentane:ether (3:1, v/v for silica and 3:2, v/v for  $AgNO_3$  argentation TLC), or the solvents indicated elsewhere in the text, with visualization using 0.2 % 2,7-dichlorofluorescein in ethanol and viewing under UV light. Liquid scintillation counting was performed in 10 ml of

cocktail consisting of 0.4 % Omnifluor (DuPont/New England Nuclear) in ethanol:toluene (3:7, v/v). Protein concentration was determined by the method of Bradford [53] using bovine serum albumin as a standard and the Bio-Rad protein assay reagent.

#### Supplementary material available

Structural assignments for taxa-4(20),11(12)-dien-5 $\alpha$ -ol (compound 5 $\alpha$ -**3**), taxa-4(20),11(12)-dien-5 $\beta$ -ol (compound 5 $\beta$ -**3**) and taxa-4(20),11(12)-diene (compound **8**) using  $^1\text{H}$  NMR, NOESY, DQF-COSY, HMQC spectroscopy and HMBC spectroscopy. Details of synthesis of [20- $^3\text{H}_2$ ]taxa-4(20),11(12)-dien-5 $\alpha$ -ol, [20- $^3\text{H}_2$ ]taxa-4(5),11(12)-diene, [20- $^3\text{H}_2$ ]taxa-4(20),11(12)-diene, [20- $^2\text{H}_3$ ]taxa-4(5),11(12)-diene and [20- $^2\text{H}_2$ ]taxa-4(20),11(12)-diene.

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